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Elevated Levels of IL-8 in Dengue Hemorrhagic Fever

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Dengue virus causes dengue fever, a mild febrile illness, and at times dengue hemorrhagic fever (DHF), a severe illness the pathogenesis of which is not fully understood. Given the crucial roles played by interleukin-8 (IL-8) as a chemoattractant cytokine and in inflammatory processes, levels of circulating IL-8 in the sera and IL-8 mRNA in the peripheral blood mononuclear cells (PBMC) were measured in 99 patients of a recent dengue epidemic that occurred in India in 1996 and in 21 normal healthy controls. Twenty-six of the patients had dengue fever (DF) and the remaining 73 were diagnosed as having different grades of DHF. All the control normal sera were negative for IL-8, so were their PBMC for IL-8 mRNA. Increased levels of IL-8 in the sera and IL-8 mRNA in their PBMC were observed in patients with severe illness of DHF grades III and IV. Only two out of 26 patients of DF and one out of 10 DHF grade I patient were positive for IL-8 and all three deteriorated to DHF grade IV within 24 hr. All six patients of DHF grade IV who died had higher serum level of IL-8 above 200 pg/ml, the highest being 5,568 pg/ml in one patient; the presence of mRNA for IL-8 was very high in all patients. A striking correlation was observed between increased levels of IL-8 and severe DHF, with greater levels in patients with increased grade of the disease and death. These results suggest that IL-8 may have an important role and may be an indicator of increasing severity of the disease and death. ***J. Med. Virol. 56:280–285, 1998.***

KEY WORDS: dengue hemorrhagic fever; interleukin-8; cytokine; dengue virus; pathogenesis

INTRODUCTION

Dengue virus infections are an important cause of morbidity and mortality in tropical and subtropical countries [Lam, 1995]. Dengue virus causes dengue fever (DF), which is typical of the painful, nonlethal fever-arthritis-rash syndrome. DF may progress after 2–5 days to a rapidly progressive, severe, life-threatening form called dengue hemorrhagic fever (DHF), characterized by increased capillary permeability, cerebral edema, altered number and functions of leukocytes, spontaneous hemorrhages, petechiae, epistaxis, increased hematocrit and thrombocytopenia, and extensive plasma leakage in various serous cavities of the body, including the pleura, pericardium, and peritoneal cavities [Reyes, 1966; Burke, 1968; Bhamarapravati, 1993]. DHF has emerged as the most important arbovirus disease in human, but its pathogenesis is not fully understood. DHF is classified into four grades, ranging from the mildest, grade I, to the most severe, grade IV, on the basis of clinical presentation and laboratory findings [Nimmannitya, 1993].

The immunopathological mechanisms leading to DHF have not been fully delineated and it is likely that cytokine cascade plays an important role. Helper T (Th) cells are differentiated into Th1 and Th2 cells, which differ from each other in their cytokine secretion profile. Th1 cells secrete interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-beta (TNF-β) and are responsible for cell-mediated inflammatory reactions, delayed-type hypersensitivity, tissue injury in infections, autoimmune diseases, and recovery from infections. Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 and are associated with B-cell antibody production and also with the increased severity and death in certain infections. Cross-regulation of the two clones is mediated by IL-10 and IFN-γ. Furthermore, TNF-α and IL-10 form an autoregulatory loop, in which TNF-α is an inducer of IL-10, and IL-10 is a downregulator of TNF-α [Powrie and Coffman, 1993;

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van der Poll et al., 1994; Perez et al., 1995; Mosmann and Sad, 1996].

Chemokines are involved in the recruitment of leukocytes to sites of infection and inflammation via a cascade of coordinated events. Chemokines synthesized during inflammation include interleukin-8, which is secreted by macrophages/monocytes, endothelial cells, fibroblasts, epithelial cells, and neutrophils, and which induces neutrophil adherence to vascular endothelium and extravasation into tissues [Matsushima and Oppenheim, 1989; Detmers et al., 1990]. It has been reported that the free radicals, nitrite and peroxynitrite, directly upregulate production of IL-1β TNF-α, IL-8, and hydrogen peroxide in macrophages, while TNF-α induces the production of IL-6, IL-8, and IL-10 [reviewed by Cerami, 1992; Merrill and Benveniste, 1996].

Elevated levels of IL-8 have been found in several viral infections. IL-8 is induced by viruses and appears in the circulation during viral infections. Increased IL-8 levels have been found in nasal aspirates from children suffering from virus-induced asthma exacerbations [Teran et al., 1997], in rhinovirus infection [Grunberg et al., 1997], in paramyxovirus infection [Friedland, 1996], in respiratory syncytial virus infection [Biswas et al., 1995], in viral otitis media [Chonmaitree et al., 1996], in chronic active hepatitis [al-Wabel et al., 1995], and in influenza virus infection [Matsukara et al., 1996]. Given the rather profound roles played by IL-8 in inflammation, binding of neutrophils to endothelial cells, and extravasation of neutrophils into tissues, it was considered useful to determine the serum levels of IL-8 and IL-8 mRNA in the peripheral blood mononuclear cells (PBMC) in patients of dengue hemorrhagic fever. A good correlation was observed between elevated IL-8 levels and the severity of dengue hemorrhagic fever.

MATERIALS AND METHODS

Patients

The present study was carried out during an extensive epidemic of dengue hemorrhagic fever that occurred in northern India from August to November 1996. The patients suffering from typical dengue-like illness admitted to the Gandhi Memorial and Associated Hospitals, Lucknow, and the Pediatrics Department of the All India Institute of Medical Sciences, New Delhi, were studied. All the patients were examined by a physician and the laboratory investigations were carried out. At the time of arrival at the hospital, the clinical presentation of every patient was recorded, Hess test was done, and the patient's hematocrit value and platelet counts were measured; the last two tests were repeated daily during their stay in the hospital. The day of the onset of fever was considered as day zero of the illness and thus the day of sample collection was calculated for each patient accordingly. Depending on the clinical presentation and the laboratory findings, they were classified as dengue fever (DF) or dengue hemorrhagic fever (DHF) grades I, II, III, or IV according to the criteria of the World Health Organization [Nimmannitya, 1993]. The grade of the illness was recorded at the time of collection of blood samples. A total of 99 cases were included in the present study; 65 of the patients were below 15 years of age, the youngest being 8 months old, the oldest being 55 years of age. Twenty-six of the patients were classified as DF, 10 as DHF grade I, 29 as grade II, 21 as grade III, and 13 as grade IV. Diagnosis of dengue virus infection was established either by virus isolation or by detection of the virus-specific IgM in the sera (data not shown). Twenty-one normal age-matched individuals, without any history of febrile or other illnesses in the last three months, were selected as controls. Sera from the patients and the controls were divided into aliquots, frozen, and stored at −60°C. Sera were transported to Kuwait on dry ice and stored at −70°C till tested.

Assay for IL-8 by ELISA

IL-8 levels in the sera were assayed by commercial sandwich ELISA kits (Immunotech, France). All tests were carried out on undiluted sera according to manufacturer's protocols. Tests were set up in duplicate and the data were analyzed by Genesis Windows Software (Labsystems, Finland). The minimum detectable concentration of IL-8 was 8 pg/ml. Because in none of the control sera was IL-8 detectable, a value of 24 pg/ml (3 times the minimum detectable value) was used as the cutoff value for designating a given serum sample as either positive or negative.

Preparation of Peripheral Blood Mononuclear Cells

Peripheral venous blood was collected in a heparinized tube from the cases of various grades of illness and normal healthy controls. The leukocyte-rich plasma was removed and the PBMC consisting of monocytes and lymphocytes were separated on Lymphoprep, density 1.077 g/ml (Nyegaard & Co., Oslo). The cells collected from the interface layer were washed three times with Hanks basal salt solution (HBSS) and counted as described earlier [Agarwal et al., 1998a, 1998b]. The PBMC thus obtained were used for extraction of RNA.

mRNA Extraction and RT-PCR

mRNA was extracted from the PBMC of the patients and the controls and used in RT-PCR for human cytokines according to the methods described [Hamida and Mustafa, 1998]. Briefly, mRNA from the PBMC (a minimum of 1×10^6 cells) was extracted by using Quick Prep Micro mRNA purification kit (Pharmacia Biotech, Sweden) according to the manufacturer's instructions. First-strand cDNA was synthesized from mRNA by using the first-strand cDNA synthesis kit (Pharmacia Biotech) according to the protocol of the kit manufacturer. PCR was carried out by using the first-strand cDNA as the template and the primers specific for the cytokine IL-8 (sense 5′-ATGACTTCCAAGCTGGCCGTG-3′ and antisense 5′-TTATGAATTCTCAGC-

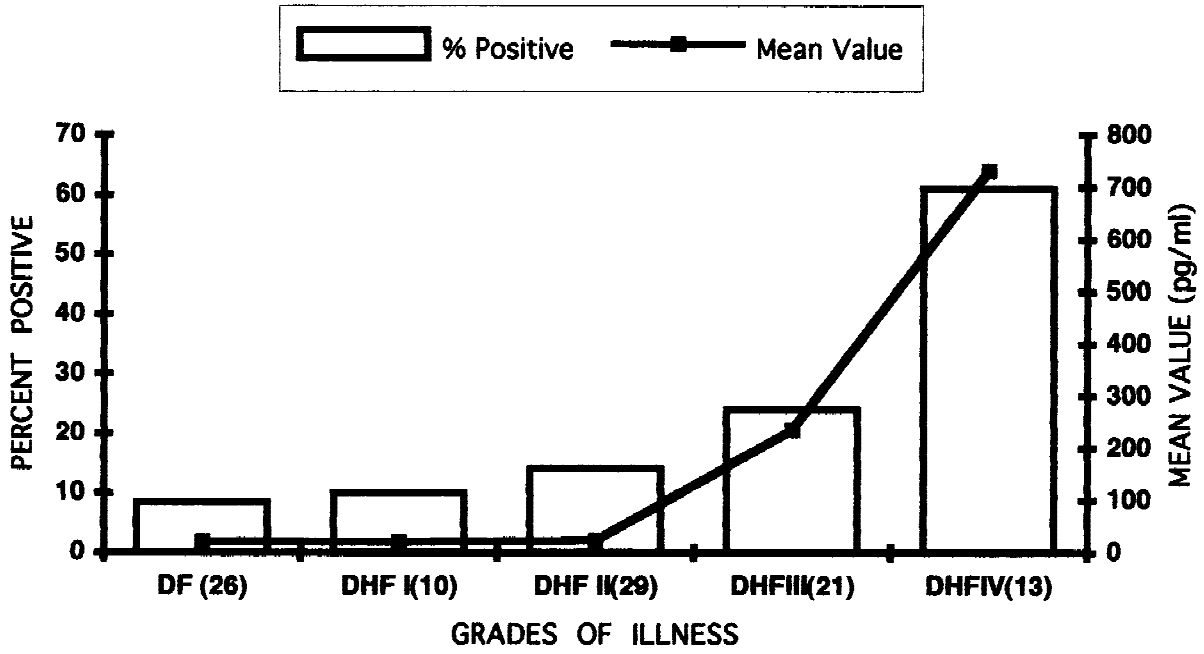

Fig. 1. Levels of serum IL-8 in dengue illness. Sera of DF and DHF (grades 1 through IV) were screened for IL-8 by ELISA. Mean values of IL-8 levels and the percentages of IL-8–positive individuals in different groups are presented.

CCTCTTCA-3′). The primers specific for the housekeeping gene, β-actin (sense 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ and antisense 5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′), were used as a positive control. The reagents of DNA PCR kit (Cetus, Perkin-Elmer, Norwalk, CT) were used in amplification reactions according to the manufacturer's instructions. PCR was undertaken for 35 cycles and the amplified DNA were analyzed by gel electrophoresis. The DNA bands for β-actin and IL-8 were size-identified by comparing with the bands of molecular weight marker DNA. In all the specimens, DNA corresponding to β-actin was amplified, suggesting that purified mRNAs were suitable for RT-PCR. A specimen was considered positive or negative for IL8 mRNA depending on the presence or absence of DNA band of expected size (corresponding to 300 bp). The data was analyzed statistically using Student's t-test. A P value of less than 0.05 was considered significant.

RESULTS

Levels of IL-8 in Sera

None of the 21 control sera had any detectable levels of IL-8. As shown in Figure 1, substantially higher amounts of IL-8 ($P < 0.001$) were detected in the sera of DHF IV, with a mean value of 643 pg/ml as compared to that in cases of DF and DHF grades I and II. DHF grade III patients had a higher mean value of IL-8 than DHF grades I and II ($P < 0.129$). Among DHF patients, 61% of grade IV and 24% of grade III were positive, while only 14% of DF patients were positive for IL-8 (Fig. 1). The conditions of the two patients of DF and one of DHF grade I, who were positive for IL-8 at the time of admission, deteriorated within 24 hr to DHF grade IV. One patient, aged 26, was in deep shock and was bleeding from all over the body when the sample was obtained on day 5 of the illness; this patient had the highest IL-8 value (5568 ± 219 pg/ml) of all patients in this study. Six of the 13 patients of DHF grade IV died, and all had profound shock with blood pressures not recordable and very high titers (above 200 pg/ml) of IL-8 (Table I).

Sera obtained from the patients at different stages of the illness were grouped between days 1 and 4, between days 5 and 8, and after day 9. From Figure 2 it is clear that while detectable IL-8 levels appeared during the first few days of infection, a similar proportion of the patients continued to be positive on days 5–8 and then declined after day 9. The data was further analyzed with respect to distribution of the patients of various grades of illness according to the time period of the illness. The data presented in Figure 3A show that none of the patients of DF had IL-8, while all the patients of DHF grades III and IV were positive (though the number was too small). During the 5- to 8-day period, 62.5% of patients of DHF grade IV were positive for IL-8 (Fig. 3B). The two patients of DF positive for IL-8 during this period had quickly deteriorated to DHF grade IV as stated above. From the ninth day onward only the patients of DHF grades III (14%) and IV (33.3%) were positive for IL-8 (Fig. 3C).

RT-PCR for IL-8 Gene Expression

The results of representative experiments for IL-8 gene expression in PBMC of control, DF, and DHF patients as determined by detection of IL-8 mRNA by RT-PCR are given in Figure 4 and the cumulated data presented in Figure 5. These results show that IL-8

TABLE I. Levels of IL-8 in Fatal Cases of DHF

ID number	Age (years)	Sex	Progression of the illness	IL-8 (pg/ml) (mean value ± SD)
967688	26	M	DF to DHF IV	5,568 ± 219
967790	5	M	DF to DHF IV	218 ± 38
967880	5	M	DHF I to DHF IV	662 ± 24
968049	11	F	DF to DHF II to DHF IV	1,059 ± 78
968256	3	F	DF to DHF II to DHF IV	1,502 ± 100
968258	5	M	DF to DHF II to DHF IV	314 ± 70

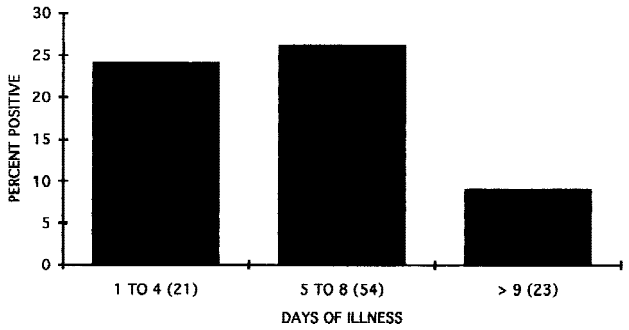

Fig. 2. Percentage of IL-8–positive individuals as a function of the stage of dengue illness. The numbers in parentheses represent the total number of patients in each group.

mRNA was clearly expressed in PBMC of majority of the cases of DHF grade IV (65%) but in none of the control subjects. In patients of DF and DHF grades I to III, IL-8 mRNA was detected in the PBMC of 15% to 30% of the cases (Fig. 5).

DISCUSSION

This is the first study to describe the levels of IL-8 in dengue illness and demonstrate an association of elevated IL-8 levels with the severity and fatal outcome of the patients of dengue hemorrhagic fever. It was observed that neither IL-8 mRNA was found in the control PBMC nor IL-8 detected in their sera. Among the DF patients, 14% had IL-8 mRNA in the PBMC and IL-8 in their sera, while 65% of the patients of DHF grade IV had mRNA in the PBMC and 61% had IL-8 in their sera. It should be noted that all six of the DHF grade IV patients who died had been in profound shock and high serum levels (>200 pg/ml) of IL-8. The intravascular coagulopathy seen in DHF [Reyes, 1966; Burke, 1968; Bhamarapravati, 1993] may be attributable, at least in part, to the effects of IL-8. Halstead [1988] proposed that the severe form of dengue illness has an immunopathological basis. Based on current knowledge of cytokine networks and their prolific and potent roles in immune reactions, it is likely that much of the pathological sequelae in dengue may be attributable to cytokines.

Among the various pathological findings in DHF are focal visceral hemorrhage, serous and bloody effusions, and retroperitoneal edema; increased vascular permeability with leakage of plasma is common. It is tempting to associate some of these changes in DHF with the presence of high levels of IL-8. It has been shown that IL-8 induces the release of lytic enzymes, platelet-activating factor, and leukotreines, and induces respiratory burst of neutrophils, all of which contribute to inflammatory reactions and infiltration of neutrophils [Baggiolini, 1995]. In young children with dengue illness, upper respiratory symptoms predominate; concurrently IL-8 is known to exacerbate airway hypersensitivity. High local IL-8 levels are also associated with pleural effusion [Loettscher et al., 1994], suggesting that the effusion into the pleura and other serous cavities in DHF may be at least partly attributable to the action of IL-8. The production of IL-8 appears to be dependent on IL-1 and TNF-α and thus the synthesis of IL-8 is dependent on cytokine networking. Elevated levels of serum IL-1β levels have been found in dengue-infected patients [Hober et al., 1993]. Interestingly, in a related study we have found high levels of serum TNF-α in DHF patients [Chaturvedi et al., 1998]; about

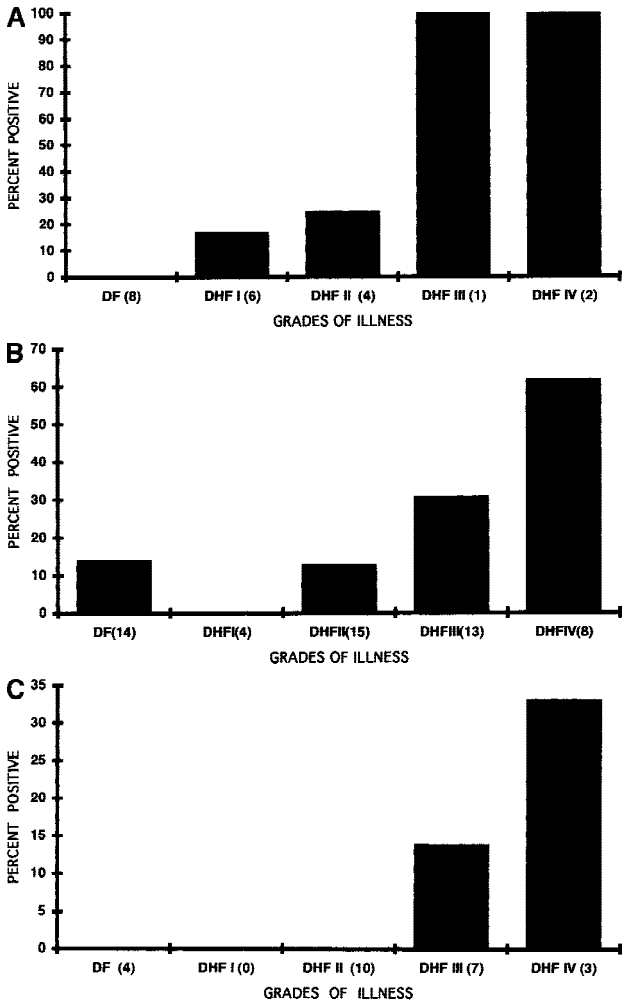

Fig. 3. Percentage of IL-8–positive individuals as a function of the grade and stage of dengue illness. **A:** During 1 to 4 days of the illness. **B:** During 5 to 8 days of illness. **C:** From the ninth day and onward of the illness. The numbers in parentheses represent the number of patients in each group.

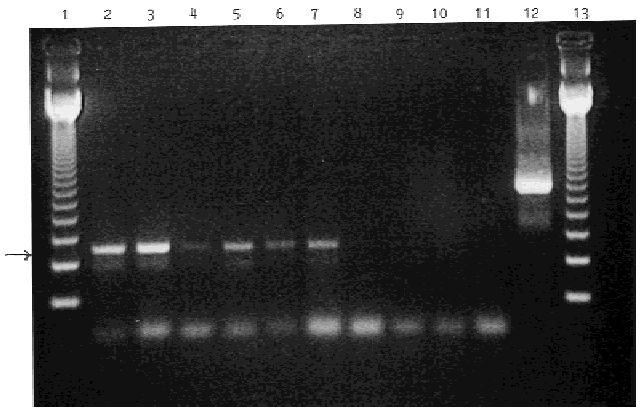

Fig. 4. Ethidium bromide-stained agarose gel of PCR products. Detection of the IL-8 mRNA (arrow corresponding to 300 bp) amplified from the peripheral blood mononuclear cells of the cases of DHF grade IV (lanes 2,3); grade III (lanes 4,5); grade II (lane 6); DHF grade I (lane 7), and DF (lanes 8,9). No IL-8 mRNA was detected in the PBMC from the normal healthy controls (lanes 10–11). The DNA bands for β-actin (lane 12) and IL-8 were size-identified by comparing with the bands of molecular weight marker DNA (lanes 1 and 13).

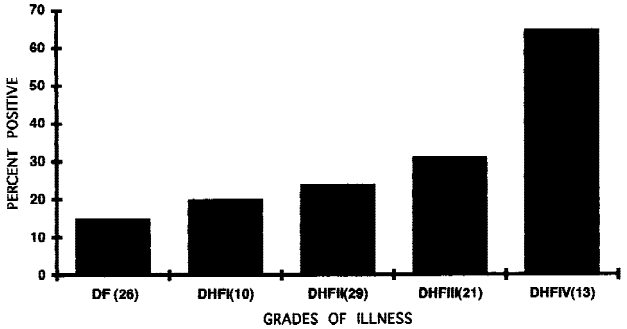

Fig. 5. Presence of IL-8 mRNA in the PBMC of the patients of DF and various grades of DHF. If no DNA band of expected size was visible on the gel, the result was recorded as negative and the presence of DNA bands of expected size was considered as positive. The percentages of the IL-8 mRNA-positive patients among those of various grades of the illness have been presented.

85% of the DHF sera tested were positive for TNF-α, with peak titers attained by day 4 of the illness. At later periods both the viremia and TNF-α disappears. Since the virus itself and TNF-α can induce IL-8 production, one may anticipate the presence of elevated levels of IL-8 especially following the attainment of the serum TNF-α peak, which is what we have observed in the present study.

Dengue virus infection induces the production, by $CD4^+$ T-cells, of a unique cytokine, the Cytotoxic Factor, in mice (mCF) and in man (hCF) [Chaturvedi et al., 1997]. The pathogenic role of mCF/hCF has been established in producing lesions in mice similar to those seen in DHF, for example, increased capillary permeability and cerebral edema [Khanna et al., 1990; Chaturvedi et al., 1991; Mukerjee et al., 1997] by inoculation of hCF purified from the sera of DHF patients. During an extensive epidemic of DHF in northern India during 1996, the presence of hCF was shown in 90% of the 333 cases, with peak amounts in the most severe cases of DHF grade IV [Chaturvedi et al., 1999]. The patients in the present study came from the same lot of the patients. Ex vivo culture of PBMC of such cases showed production of hCF by $CD4^+$ T-cells[Agarwal et al., 1998a, 1998b]. A shift from Th1-type to Th2-type response was observed in these patients correlating with increasing severity of the illness, thus indicating a possible role for Th2-type response in the pathogenesis of DHF [Chaturvedi et al., communicated]. The mechanism of action of mCF/hCF has been delineated; they selectively kill Th cells, $H\text{-}2A^+$ macrophages, and the cells capable of liberating histamine via production of nitrite and respiratory burst, leading to the formation of peroxynitrite and resulting in apoptosis of the target cells and increased vascular permeability, which causes plasma leakage and hemorrhages that produce DHF [Khanna et al., 1990; Chaturvedi et al., 1991; Mukerjee and Chaturvedi, 1995; Mukerjee et al., 1996; 1997; Misra et al., 1996].

Increased vascular permeability and its consequences in dengue hemorrhagic appear to be a multifactorial phenomenon in which IL-8 is an important participant. Quick deterioration of the condition of two cases of DF and one case of DHF grade I who were positive for IL-8, and a consistent correlation of high IL-8 titer with the fatality, together show that determination of IL-8 in a patient may be a useful indicator of a serious outcome of dengue illness.

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